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Sublethal Effects of Chronic Oil Exposure on the Intertidal Clam 'Macoma balthica'

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16. ABSTRACT

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Sublethal Effects of Chronic Oil Exposure on the Intertidal Clam *Macoma balthica**

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Abstract

When exposed to Prudhoe Bay crude oil in flowing seawater for 180 days, the small intertidal clam *Macoma balthica* showed behavioral, physical, physiological and biochemical changes. At a high concentration of oil in seawater (3.0 mg l^{-1}) burrowing rate decreased, respiration rate increased, growth was inhibited, and very high mortalities resulted. The lowest concentration of oil in seawater (0.03 mg l^{-1}) inhibited growth and caused reabsorption of gametes. One group of adverse oil effects which was related to sluggishness and disorientation of the clams appeared after a week of exposure to oil; another group related to a negative energy balance was not observed until 60 days. We conclude that chronic exposure of *M. balthica* to oil-in-seawater concentrations even as low as 0.03 mg l^{-1} will, in time, lead to population decreases.

Introduction

One of the more noticeable effects of severe oil pollution in the sea is mass mortality (*inter alia*: Krebs and Burns, 1977). However, mortality, although easy to measure, is the grossest of all effects of oil pollution. More subtle sublethal effects of oil pollution include changes in respiration (Hargreave and Newcombe, 1973; Eldridge *et al.*, 1977), cellular morphology (Gardner *et al.*, 1975), reproductive development (Berdugo *et al.*, 1977; Byrne and Calder, 1977), feeding (Atema and Stein, 1974; Morton and Wu, 1977), growth and maturation (Griffin and Calder, 1977; Keck *et al.*, 1978), behavior (Hargreave and Newcombe, 1973; Donahue *et al.*, 1977), and biochemistry (Manwell and Baker, 1967; Davavin *et al.*, 1975). To date, only a few such parameters have been measured for any single species.

We report here an experiment which examined mortality and behavioral, physical, physiological, morphological and biochemical effects of 180 days' petroleum exposure of the bivalve mollusc *Macoma balthica*. The intent of this work was to investigate simultaneously a variety of biological parameters, the alteration of which might indicate oil-induced stress. By exposing separate groups of test clams to three oil-in-water concentrations (nominally 0.03, 0.3 and 3.0 mg l^{-1} of Prudhoe Bay crude oil) we obtained information about the degree of stress as a function of oil concentration.

Macoma balthica is abundant in Alaskan and other subarctic and temperate intertidal environments with fine-grained sediments. As both a deposit-feeder and a filter-feeder, this clam is probably exposed to both stranded and dispersed petroleum in nature. A field population of this species has been shown to be affected by oil (Shaw *et al.*, 1976). *M. balthica* is also a food for many migratory shore birds (Heppleston, 1971; Bagge *et al.*, 1973), and thus has the potential to pass petroleum hydrocarbons up the food web.

Materials and Methods

Macoma balthica were collected and maintained at Seward, Alaska (149°27′W; 60°06′N), in petri dishes filled to a depth of 2.5 cm with sand immersed in continuous-flow exposure tanks which measured 110 cm x 50 cm x 36.5 cm deep (Clement *et al.*, 1980). Fresh seawater flow was maintained at approximately 3.0 l^{-1} min. After 1 week, clams which remained on the surface were removed and replaced until all individuals had buried themselves. On 14 June 1977, following a 2-week acclimation period, the clams were exposed continuously for 6 months to dispersions of Prudhoe Bay crude oil in

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seawater. Detailed characterizations of these oil-in-water dispersions and the resulting concentrations of hydrocarbons in *M. balthica* at various times during and after exposure are reported in the preceding study (Clement *et al.*, 1980). The average levels of petroleum hydrocarbons in each of 4 treatments were, approximately: control, 0.0 mg l^{-1}; low oil, 0.03 mg l^{-1}; medium oil, 0.3 mg l^{-1}; high oil, 3.0 mg l^{-1}. Water temperature (7.0 ° to 9.1 °C) and salinity (31.6 to 34.0 ‰) were essentially the same for all treatments. Tanks were cleaned periodically and dead individuals were counted and removed. The experimental clams were fed for an average of 4 to 6 h a day by pumping diatom-rich water (mean phytoplankton density 7,300 cells ml^{-1}) into the tanks from a nearby "upwelling pond" (Nevé *et al.*, 1976). Feeding began on 9 July and was stopped on 21 November 1977.

To determine relative burrowing rates, 100 clams (6 to 11 mm in length) from each treatment were placed on top of sand in a Pyrex dish (25.3 cm x 15.2 cm x 4.4 cm deep) set 4 cm below the surface of the water of the exposure tanks. At timed intervals over the next 24 h, the numbers of unburied clams were recorded. The same clams were utilized for each burying experiment. Results were expressed as the percentage of the clams which remained unburied after 1 h. Wet and dry weights were determined on approximately 30 clams for each treatment. Wet weights were obtained from depurated and shucked clams. Dry weights were measured after lyophilization for 12 to 24 h.

One hundred clams from each treatment (10 clams per petri dish) were selected to monitor growth and condition. These clams (10.5 mm long, standard deviation = 0.15 mm) were left undisturbed from 14 June to 12 October 1977. The measurements taken at the beginning and the end of the experiment were shell length and dry tissue weight. Dry weight was obtained by drying the tissue in an oven at 80 °C until a constant weight was reached. A condition index relating dry weight to shell length was calculated by:

$$\text{Condition index} = \frac{\text{dry weight (g)}}{\text{shell length (mm)}} \times 100.$$

Macoma balthica at Day 120 of the experiment were depurated and then placed in formalin. The gonads were cut out and dehydrated in ethanol (Davenport, 1960). The tissues were cleared in xylene and embedded in paraffin. Sections of 20 μm thickness were cut on a microtome, stained with Ehrlich's acid alum hematoxylin, and then examined under a microscope to determine the condition of the gonad tissue.

Oxygen uptake by *Macoma balthica* was measured in a smaller version of the modified Scholander respirometer described by Steen and Iversen (1965). The air volume was 15 ml and the water volume 100 ml, which allowed changes in air volume of 1 μl to be detected. The respirometers without clams were stable to ± 2 μl O_2 h^{-1} after equilibration. For each respiration-rate measurement, 2 or 3 clams were removed from the exposure tanks before feeding, screened from the sand, returned to the exposure tanks for 2 h, and then placed in the respirometer chambers. Respirometers were allowed to equilibrate for 1 h, and then oxygen uptake was measured at 30 min intervals for 3 to 4 h at 10 °C. After the final measurement, clams were removed from their shells, weighed, lyophilized, and reweighed. Respiration rate is expressed as μl O_2 consumed (standard temperature and pressure) per mg dry weight per hour.

Clams were sampled for various biochemical assays by removing randomly selected petri dishes and washing the clams onto a screen. The clams were then returned to the tanks from which they had been removed to depurate for 24 h, during which time no food was administered (except that clams to be assayed for enzymes were homogenized at once). After 24 h depuration, the clams were shucked, weighed, lyophilized, weighed dry, and stored in desiccators at −20 °C until assayed. Total protein and total carbohydrate were measured in individual clams. Lyophilized clams were soaked in 1.0 ml of cold distilled water for 15 to 30 min and then homogenized at 0 °C in a 5.0 ml glass-Teflon tissue-homogenizer at 2250 rpm for 25 to 30 sec. The homogenate was made up to 4.0 ml with distilled water and stored frozen until assayed. Protein in the crude homogenate was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin (BSA, Sigma Chemical Co.) as the standard. Total carbohydrate in the crude homogenate was assayed by the anthrone procedure, with glucose as a standard (Spiro, 1966).

Total lipid was also determined for individual clams. To the lyophilized clam tissue was added 100 μl of distilled water on ice. After 15 to 20 min, 2.0 ml of benzene:menthanol (2:1 v/v) were added, and the clam was homogenized in a 5.0 ml glass-Teflon tissue-homogenizer at 2000 rpm for 30 sec at 3° to 4 °C. The homogenizer was rinsed with an additional 1.0 ml of benzene: menthanol. The extract was washed with 1.0 ml of 0.9% NaCl in water and centrifuged. The resulting upper lipid layer was transferred, and the water phase was re-extracted with 1.0 ml of benzene. The combined lipid extract was washed once more with 1.0 ml 0.9% NaCl and the water phase was again back-extracted with 1.0 ml of benzene. All lipid phases were combined in a tared vial. The contents of the vials were evaporated to dryness under N_2, and the vials were reweighed to obtain the total lipid weight.

Ribonucleic acid (RNA) was separated from deoxyribonucleic acid (DNA) by a modified Schmidt-Thannhauser procedure suggested by Munro and Fleck (1966). RNA was determined by its absorbance at 260 nm with calf liver RNA (Sigma Chemical Co.) as a standard. DNA was extracted in hot (70 °C) 1.2 N $HClO_4$, and estimated using diphenylamine, according to the procedure of Giles and Meyers (1965). It was found unnecessary to correct for turbidity by measuring the absorbance at 700 nm. Calf thymus DNA (Sigma Chemical Co.) served as a standard.

To prepare a crude homogenate for enzyme assays, 10 clams from each treatment were screened from the

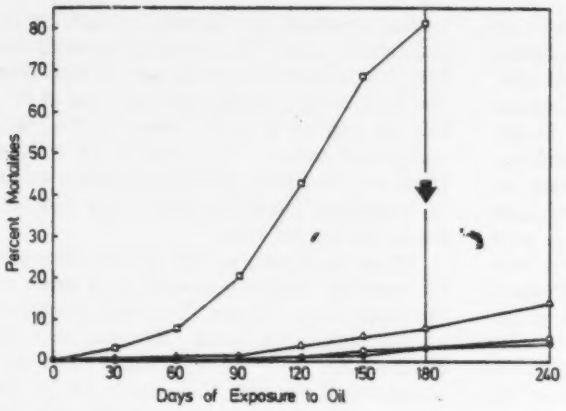

Fig. 1. *Macoma balthica*. Cumulative mortalities for the various oil treatments during 6 months oil exposure. Filled circles: controls; open circles: 0.03 mg l^{-1}; triangles: 0.3 mg l^{-1}; squares: 3.0 mg l^{-1} oil; arrow indicates end of oil exposure

sand and placed in cold (2° to 4 °C), fresh seawater. The clams were shucked and placed in a 15 ml conical centrifuge tube. Three milliliters of cold "homogenization buffer" (0.25 M sucrose, 1 mM ethylenediaminetetraacetate, EDTA; pH 7.0) were added, and the contents mixed and centrifuged. The supernatant was decanted, which removed most of the unabsorbed oil droplets from the clams. The clams from such treatment were then homogenized in 10.0 ml of the homogenization buffer on an ice bath at top speed for two 10-sec bursts in a Virtis "45" homogenizer. The homogenate was passed through nylon gauze into a 15.0 ml glass-Teflon tissue-homogenizer. The solution was further homogenized at 1000 rpm for 3 strokes and stored frozen at −20 °C in aliquots for enzyme assays.

For total ATPase the assay mixture contained in a final volume of 1.0 ml: 50 mM imidazole · HCl (pH 7.5), 4 mM $MgCl_2$, 0.5 mM Na_2 EDTA, 20 mM NaCl, 20 mM KCl, 3 mM adenosine triphosphate (ATP) and 0.100 ml of the crude homogenate. To measure the Mg^{++}-dependent ATPase, KCl and NaCl were omitted, and ouabain was added at a final concentration of 2 mM. Na^+-K^+-ATPase activity was taken as the difference between the total ATPase and the Mg^{++}-ATPase activities. Incubations were carried out at 20 °C for 10 min, and stopped with 2.0 ml of cold ascorbate:trichloroacetic acid (TCA) (2.5%:12.5% w/v). Inorganic phosphate (Pi) was measured by the method of Baginski *et al.* (1974). Results were expressed as nm Pi released min^{-1} mg^{-1} protein.

The 5′-nucleotidase was assayed essentially as described by Aronson and Touster (1974), with the following modifications. A volume of 0.1 ml of crude homogenate was assayed in 0.5 ml total. The reaction was run at 30° for 15 min and stopped with 1 ml of ascorbate:TCA (2.5%:12.5% w/v). Inorganic phosphate was measured by the method of Baginski *et al.* (1974). Results were expressed as nm Pi released min^{-1} mg^{-1} protein.

The substrate used to assay phosphodiesterase was thymidine-5′-monophospho-p-nitrophenol ester (Sigma Chemical Co.). The enzyme was assayed according to the method of Aronson and Touster (1974). A total volume of 0.5 ml was used, including 50 µl of the crude homogenate. Assays were run at 30 °C for 15 min. Results were expressed as changes in absorbance at 400 nm min^{-1} mg^{-1} protein. *In vitro* effects of crude oil and other hydrocarbons on Mg^{++}-ATPase were assayed by mixing (vortex) measured amounts of the hydrocarbons (1 to 100 µl) with 10 ml of crude clam homogenate. Aliquots of these mixtures were then assayed for enzyme activity.

Results

There were significant mortalities of *Macoma balthica* in the oil treatments compared to the controls. The total cumulative mortality in the control tanks after 6 months was about 3.1% (Fig. 1). The clams in 0.03 mg l^{-1} had a similar mortality of 3.2% after 6 months. In the 0.3 mg l^{-1} treatment, mortalities reached 8.0% by Day 180 and were significantly different from the controls by Day 120 ($P < 0.01$, arcsine t-test). The clams exposed to 3.0 mg l^{-1} began to die by Day 30 (2.9%, $P<0.001$). By Day 180, mortality had reached 81%.

During feeding periods, the controls and the clams in 0.03 mg l^{-1} would extend their siphons and begin very active suspension- and desposit-feeding behaviour (Brafield and Newell, 1961). The clams in 0.3 mg l^{-1} appeared to feed less actively, and those in 3.0 mg l^{-1} very sluggishly, rarely extending their siphons at all. Survivors exhibited the foregoing behaviours throughout the 6 months of exposure to oil.

Initially, all clams were buried in the sand of their petri dishes. During the first week of exposure, clams in both the high (3.0 mg l^{-1}) and the medium (0.3 mg l^{-1}) oil exposures burrowed out of the sand to the surface. Only approximately 10% of the clams emerged on the surface of the sand. By Day 16, almost all of the clams in the medium exposure had reburied themselves. The clams exposed 3.0 mg l^{-1}, however, tended to remain at the surface of the sand with progressively more surfacing throughout the experiment. Exposure to 3.0 mg l^{-1} for 180 days caused over 90% of the surviving clams to burrow to the surface. It was not until Day 90 that clams in 0.3 mg l^{-1} began to burrow to the surface in significant numbers ($P < 0.01$). We also noted that clams which were unburied at 3.0 mg l^{-1} were less able to orient themselves with respect to the surface of the sand. These clams would extrude their feet which would probe in various directions, but were unable to penetrate the sand; thus, they appeared unable to rebury themselves once they had come to the surface.

Fig. 2 summarizes the results of experiments in which clams were removed from sediment, placed on sand and the number remaining unburied after 1 h determined. On the first day of exposure to 3 mg l^{-1}, clams showed a significant decrease in their burrowing rate compared to

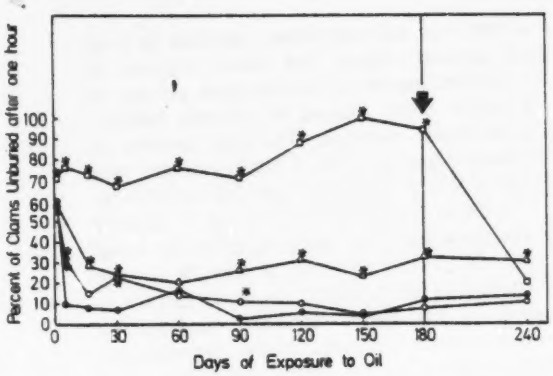

Fig. 2. *Macoma balthica*. Percentage clams remaining unburied 1 h after being placed on sand surface. Asterisks indicate values significantly different from controls at $P<0.01$ (except for 3.0 mg l^{-1} at Day 0 where $P<0.02$, and 0.03 mg l^{-1} at Day 90 where $P<0.05$). Arcsine t test was used to evaluate data. Filled circles: controls; open circles: 0.03 mg l^{-1}; triangles: 0.3 mg l^{-1}; squares: 3.0 mg l^{-1} oil; arrow indicates end of oil exposure

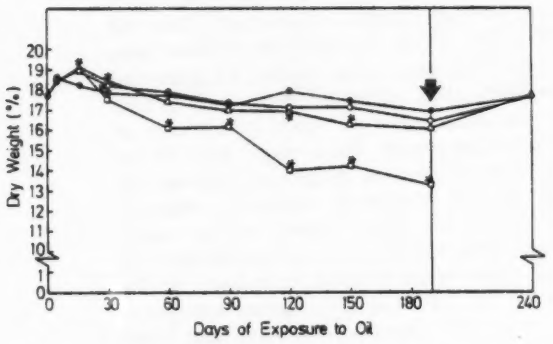

Fig. 3. *Macoma balthica*. Percentage dry weight of clams throughout 6 months oil exposure. Asterisks indicate values significantly different from controls. Filled circles: controls; open circles: 0.03 mg l^{-1}; triangles: 0.3 mg l^{-1}; squares: 3.0 mg l^{-1} oil; arrow indicates end of oil exposure

the controls ($D < 0.02$, arcsine t-test). This decrease became even greater as the experiment progressed ($P < 0.001$ at Days 5-180). Clams at 0.3 mg l^{-1} also showed significant effects from Day 5 onward. Day 60 was anomolous because of a power fallout and subsequent cessation of the water flow for 12 h; many clams in the control tanks burrowed to the surface at this time, probably due to decreased dissolved-oxygen levels. Clams at 0.03 mg l^{-1} oil were also slightly affected in comparison to the controls.

The initial wet tissue weight for the clams was about 153 mg per clam. During the course of the oil-exposure, this value decreased in all four treatments. The control values dropped to 118 mg per clam by Day 180. The clams at 0.03 mg l^{-1} showed little change in wet weight until Day 150, when a large decrease took place. The clams at 0.3 mg l^{-1} oil had values similar to the controls up to Day 120, after which the treated clams showed a greater loss in wet weight. The most marked decrease was in the 3.0 mg l^{-1} treatment, where the wet weight began to decrease immediately. By Day 180 these clams averaged only 99 mg wet weight each. Dry weights also showed decreases over the 6 month period. Here again the 0.3 mg l^{-1} and the 3.0 mg l^{-1}-treated clams showed a greater decrease in weight compared to the controls and the 0.03 mg l^{-1} -treated clams.

When the dry weights are divided by the wet weights to correct for size differences, a clearer pattern emerges (Fig. 3). The controls and 0.03 mg l^{-1} clams maintained a dry:wet ratio of around 17 to 18% throughout the exposure period. The clams at 0.3 mg l^{-1} initially had higher values but by Day 120 were significantly lower than the controls. The clams in the 3.0 mg l^{-1} treatment had significantly lower dry to wet weight ratios than the controls for most of the data points measured.

Shell lengths were measured at the beginning of the experiment and after exposure to oil for 4 months (Table 1). Although the experiment took place during a time when slow growth was expected in the light of work by Beukema and deBruin (1977), the control clams showed an average increase of 0.50 mm in shell length. Clams from all oil treatments showed signifi-

Table 1. *Macoma balthica*. Growth and condition index after 4 months exposure to oil in seawater. SE: standard error around the mean. Numbers of individuals investigated are in parentheses

Treatment	Day	Mean shell length (mm ± SE)	Condition index[a] (± SE)
Pre-experiment	0	10.50 ± 0.015 (100)	0.165 ± 0.0007 (100)
Control	120	11.00 ± 0.028 (99)	0.142 ± 0.0012 (99)
0.03 mg l^{-1}	120	10.60 ± 0.017*(95)	0.149 ± 0.0007*(90)
0.30 mg l^{-1}	120	10.46 ± 0.094*(98)	0.138 ± 0.0008*(80)
3.0 mg l^{-1}	120	10.51 ± 0.054*(47)	0.109 ± 0.0010*(47)

[a] condition index = $\frac{\text{dry weight (g)}}{\text{shell length (mm)}} \times 100$

*values significantly different from controls (Day 120) at $P < 0.01$ (Students's t-test)

Table 2. *Macoma balthica*. Oxygen consumption rates (µl O_2 mg^{-1} dry weight h^{-1}) after exposure to oil in seawater. Two determinations were made of control and 3.0 mg l^{-1} clams after 120 days oil exposure, 4 of control and 3.0 mg l^{-1} clams after 150 days oil exposure, and 5 of 0.03 and 0.3 mg l^{-1} clams after 150 days oil exposure

Treatment	N	Oxygen consumption (± SE)	Mean dry weight per clam (mg ± SE)
Control	6	0.406 ± 0.027	30.7 ± 2.4
0.03 mg l^{-1}	5	0.477 ± 0.036	30.1 ± 2.8
0.3 mg l^{-1}	5	0.486 ± 0.034*	24.2 ± 1.6
3.0 mg l^{-1}	6	0.578 ± 0.039**	17.1 ± 3.9**

*Values significantly different from controls at $P < 0.05$

**Values significantly different from controls at $P < 0.01$ (Student's *t*-test)

antly less growth. A condition index was calculated for he clams after 4 months exposure to the oil. This time eriod was chosen rather than 6 months because of a lecrease in algal numbers in the upwelling pond used or feeding the clams. Table 1 shows that the condition ndex for the control clams was lower than that for lams exposed to 0.03 mg l^{-1}, but was significantly igher than those for the 0.3 and 3.0 mg l^{-1} clams.

Stages in the female reproductive cycle of *Macoma althica* could be recognized from histological examin- tion of gonads removed from individuals after 120 days xposure to oil Although the number of observations as small, there appeared to be some digestion of ova by moebocytes in all oil treatments, but none in the ontrols. There were no obvious differences between reated males and controls, and the sex ratio was lose to unity for all groups.

Respiration rates of *Macoma balthica* after 120 and 50 days are shown in Table 2. Significant increases in he respiration rate were observed in clams at the 0.3 nd 3.0 mg l^{-1} exposure levels. The slight increase in ate at the lower exposure level (0.03 mg l^{-1}), although ot statistically significant, is difficult to interpret ecause of the large relative standard error.

There was no definite trend for values of protein ontent as percentage of dry weight during the exposure eriod. Control values ranged from 44.4 to 51.3% of the ry weight. At Day 30 (and Day 60 for the 0.3 mg l^{-1} reatment), all the oil-treated clams displayed significantly ower values of percent protein compared to the controls, ut appeared to compensate for this deficiency during he remainder of the experiment. The absolute values of rotein for all the treatments, in mg protein per clam, lid decrease in the same fashion as the corresponding dry eight. Total carbohydrate for control clams was initially 2.1% of their dry weight and gradually dropped to 3.94% on Day 150. By Day 60, those clams exposed to 3.0 mg l^{-1} had a carbohydrate content of only 6.16%; his value remained nearly constant for the rest of the xperiment. The clams exposed to 0.3 mg l^{-1} also showed a slower, but still significant, decrease in total carbohydrate content. Again, the clams exposed to 0.03 mg l^{-1} showed essentially an identical pattern to the control group. Lipid content of *Macoma balthica* exhibited considerable variation. The lipid content of the control clams began at 9.2% of the dry weight, rose to a peak of 12.0% at Day 90, then fell to 7.0% by Day 180. The clams from 0.03 and 0.3 mg l^{-1} showed essentially the same patterns, being significantly lower than the controls of Day 30, and at Day 90 for the 0.3 mg l^{-1} treatment ($P < 0.05$). The lipid pattern in clams exposed to 3.0 mg l^{-1} generally displayed an increased lipid content compared to the controls, and the increase at Day 120 was highly significant ($P < 0.005$, 15 degrees of freedom) and Day 180 ($P < 0.01$, 12 DF, Student's *t*-test).

RNA values for all the treatments were virtually the same throughout the experiment. There was an initial drop of RNA from 34 µg RNA mg^{-1} dry tissue weight to about 24 µg mg^{-1} by Day 30, after which the values remained essentially constant. The measured amount of DNA mg^{-1} dry tissue weight showed a bimodal distribution. One group of values ("low" DNA) fell within 10 to 30 µg DNA mg^{-1}, and the other group ("high" DNA) had much higher values ranging from 90 to 120 µg DNA mg^{-1}. Very few values fell between these two groups. Based on histological studies, we concluded that the low DNA clams represent females, and the high values represent males, since we found no evidence of clams with immature gonads. The average values for the "low" DNA clams showed no differences between the control and the oil treatments, indicating relatively little effect of oil on DNA content in the clams. However, there appears to be a general trend in all of the treatments to higher values of DNA from Day 0 to Day 180. The pattern for the "high" DNA values is similar to that of the "low" DNA clams, with no significant differences between the controls and any of the oil treatments.

Specific activity of Na^+-K^+-ATPase was not affected by oil treatment (Fig. 4). However, Mg^{++}-ATPase in the control clams (Fig. 4) showed a general increase in activity throughout the experiment, although there was no corresponding increase in clams exposed to oil. Linear regressions were fitted to the data points and an analysis of covariance was calculated for the treated clams compared to the controls. The 0.03 mg l^{-1} treatment showed no significant effect of oil, but the 0.3 and 3.0 mg l^{-1} treatments had slopes significantly different from the control, with $P < 0.05$ and $P < 0.01$, respectively. This corresponds to an inhibition by Day 180 of about 24% at 0.3 mg l^{-1} and 41% at 3.0 mg l^{-1} oil. The level of activity of phosphodiesterase in the control clams remained constant during the experiment, but exposure to oil was correlated with an increase in specific activity that was significantly greater (by 55%) than the control in the 3.0 mg l^{-1} treatment ($P < 0.01$). Since the specific activities of the 5′-nucleotidase showed no correlation with respect to day of exposure, the values for each treatment were pooled, averaged, and the means tested for significance against the controls using a two-tailed

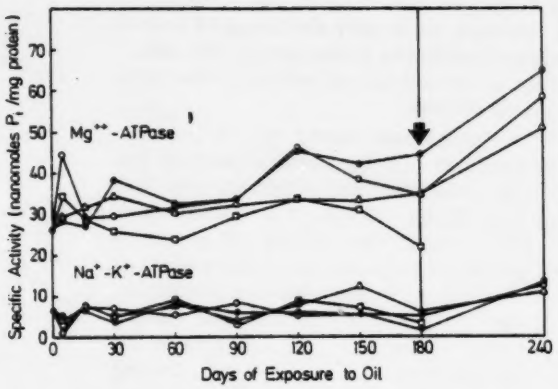

Fig. 4. *Macoma balthica*. ATPase activity during 180-day oil exposure. Filled circles: controls; open circles: 0.03 mg l^{-1}; triangles: 0.3 mg l^{-1}; squares: 3.0 mg l^{-1} oil; arrow indicates end of oil exposure

Table 3. *Macoma balthica*. *In vitro* effects of crude oil and components of crude oil on Mg^{++}-ATPase from crude homogenates of clams prepared and assayed according to procedure in "Materials and Methods". Test hydrocarbons were added after preparation of the crude homogenate in amounts specified in table, water was added to obtain a final volume of 1.1 ml. These solutions were then vortexed to mix the hydrocarbons with the crude homogenate. From this 100 µl were assayed for Mg^{++}-ATPase activity. Controls contained 100 µl of water per ml crude homogenate

Hydrocarbon treatment	Amount added per ml crude homogenate (µl ml^{-1})	% inhibition compared to controls
Prudhoe Bay crude oil	10	20
	20	22
Benzene	1	0
	10	31
	100	50
Toluene	1	0
	10	23
	100	60
Tetradecane	1	13
	10	16
	100	38

t-test. For Days 5 through 90, the 3.0 mg l^{-1} treatment displayed significantly higher values at $P < 0.02$ (DF = 8). For Days 5 to 180, the 0.03 and 0.3 mg l^{-1} treatments had significantly higher values than the controls at $P < 0.05$ (DF = 15). By Day 180, all the values from all the treatments were identical. Results from the *in vitro* enzyme assays on the crude clam homogenate indicate that both crude oil and pure hydrocarbons have the ability to inhibit Mg^{++}-ATPase activity (Table 3). The data suggest that aromatic hydrocarbons are more effective inhibitors of Mg^{++}-ATPase than are unbranched alkanes. Toluene was most effective, causing a 60% inhibition at a concentration of 100 µl per ml of crude homogenate.

After 180 days of exposure to oil, the clams were allowed to remain in the tanks with fresh flowing seawater. Most of the same parameters were measured after two months of recovery. This is noted as Day 240 on the figures. In the 3.0 mg l^{-1} treatment, there were only enough clams remaining after Day 180 for the burrowing experiment; therefore, recovery of the other parameters was investigated only for the control and the two lower oil concentrations. In general, the clams showed only partial recovery after 2 months in clean seawater. The rate of burrowing of the 3.0 mg l^{-1} treated clams increased significantly, but the clams at 0.3 mg l^{-1} remained at the Day 180 level (Fig. 2). The number of clams unburied continued to increase for the 0.3 mg l^{-1} treatment. Mortalities also continued to increase in the 0.3 mg l^{-1} treatment (Fig. 1). Wet weights continued to decline for the oil-treated clams. The dry weights, however, leveled off, which caused the dry to wet weight ratios to regain the same value as for the controls (Fig. 3).

Protein values for the oil-treated clams dropped significantly below the controls at Day 240. Total carbohydrate also continued to decline significantly in the 0.3 mg l^{-1} clams, dropping to 5.13%. Lipid levels showed a significant decrease in the 0.03 mg^{-1} clams compared to the controls ($P < 0.05$), but there were no significant differences between the 0.3 mg l^{-1} clams and controls. RNA and DNA values were not significantly different, except for the 0.03 mg l^{-1} treatment where there was a significant decrease in the relative amount of "low" DNA ($P < 0.05$). The phosphodiesterase activity of the 0.3 mg l^{-1} -oiled clams appeared to remain higher than the control value. Likewise, the Mg^{++}-ATPase activity, although increased, was still below the value for the controls (Fig. 4).

Discussion

Table 4 summarizes the results of exposing *Macoma balthica* to 6 months of Prudhoe Bay crude oil in seawater. Before comparing the effects of the oil treatment on the clams, it is important to establish that the clams in the control treatment were not themselves subjected to an unusual stress in the laboratory. The low number of mortalities (3%) in the control clams during the 6 months of experimentation suggests that the stress level was not high. However, changes in wet and dry weights, condition factor, and carbohydrate content indicate a lack of sufficient energy input for the control clams, causing their reliance on energy reserves.

In the laboratory, tides were absent. Moreover, in our experiments the clams were placed in sand which had a larger average grain size than the natural sediment from which they had been taken; also, the sand in the laboratory was not periodically distributed by tidal action such as occurs in nature. Most of the sand grains

ble 4. *Macoma balthica*. Summary of effects of 6 months posure to oil in seawater on various parameters. Normal, normal, decreased, increased, indicate levels compared to ose of control clams

rameters measured	Level of oil in exposure tanks (mg l^{-1})		
	0.03	0.3	3.0
ortality	normal	increased	increased
havior			
general activity	normal	decreased	decreased
burrowing rate	normal	decreased	decreased
% unburied	normal	increased	increased
feeding activity	normal	decreased	decreased
ysical			
wet weight	normal	decreased	decreased
dry weight	normal	decreased	decreased
dry: wet weight	normal	decreased	decreased
growth	decreased	decreased	decreased
condition index	increased	decreased	decreased
orphology			
gonads	reabsorbed	abnormal	abnormal
espiration rate	normal	normal?	increased
ochemical	normal	normal	normal
protein	normal	decreased	decreased
lipid	normal	normal	increased
RNA	normal	normal	normal
DNA	normal	normal	normal
Na^+-K^+-ATPase	normal	normal	normal
Mg^{++}-ATPase	normal	normal	decreased
phosphodiesterase	normal	normal	increased
5′-nucleotidase	increased?	increased?	increased?

the experimental substrate were larger than those *lacoma balthica* is reported to ingest during deposit-eding (Gilbert, 1977) and, in addition, the main food f the laboratory clams was a suspension of algae from ie upwelling pond; such algae do not appear to be a ivourable diet for *M. balthica*. Even though histological ections on control clams at Day 120 revealed full guts, ry weights were declining.

Beukema and de Bruin (1977) described seasonal ariations in physical and chemical composition of *lacoma balthica* in the Dutch Wadden Sea, where rowth takes place mainly during April through June. or the rest of the year the rate of growth is nearly zero, nd the ash-free dry weight falls to 55% of the June alue by the end of December. The condition index, herefore, also drops correspondingly during the same eriod. The experiments in the present investigation ere performed from June to December 1977, which oincides with the period of low growth and decreased eight found in clams in the Wadden Sea. It seems not nlikely to us that the control clams in our experiment ere acting in an essentially normal seasonal pattern.

Table 4 shows that the higher oil concentrations aused many more disturbances in the clams than the low oil concentration. The clams exposed to 0.03 mg l^{-1} showed significant differences from the controls in growth and condition index, they also appeared to have greater histological anomalies of the gonads. But, otherwise, the clams at the low oil concentration were little different from the controls.

The slow burrowing rate caused by oil exposure appears to be the result of either a general narcotization as described by other investigators (Morton and Wu, 1977; Percy and Mullin, 1977), or a disorientation mechanism. The clams in the 3.0 mg l^{-1} treatment would make efforts to bury themselves by protruding their feet, but they seemed to lack a sense of orientation. It is possible that along with being narcotized, causing sluggish movements of the foot, the statocysts in the clams were affected by the oil exposure, causing disorientation with respect to gravity and thus retarding their burying rate. In nature, specimens of *Macoma balthica* surface periodically, move about and then rebury themselves; this behaviour pattern may permit location of a better site for feeding (Brafield and Newell, 1961) or may result from infection by trematodes (Hulscher, 1973). Although we observed trematode infection in some clams, we did not investigate its frequency or relation to other parameters. Whatever the reason for surfacing, if reburial is inhibited, the clams expose themselves longer to predation and the chance of being transferred to an area of unsuitable substrate by tidal action.

The same hazards caused by slow reburial also confront clams which burrow to the surface of the sediment more frequently. Taylor and Karinen (1977) reported that exposure of *Macoma balthica* to oil caused them to come to the surface. Shaw *et al.* (1977) found that their tendency to come to the surface after 3 days exposure to an oil slick seemed to be a function of sediment depth. Individuals in 3.5 cm of sediment remained buried after oil exposure, but those in only 1.0 cm of sediment did come to the surface in significant numbers. The sediment in the petri dishes in the present experiments was 2.5 cm deep and again the clams came to the surface in significant numbers. However, in order to extend the result of Shaw *et al.* (1977), we placed groups of 10 clams in large beakers filled with sand to a depth of about 9 cm. These beakers were placed in the tanks along with the petri dishes. Although the clams were slower to surface in the beakers, after 150 days of oil exposure, 8 of the 10 clams in the beaker in the high concentration of oil (3.0 mg l^{-1}) had surfaced. This indicates that, under conditions of chronic oil pollution, *M. balthica* will eventually burrow up and out of the sediment. Further, individuals on the surface will be slower to rebury themselves (Fig. 2).

The condition index at Day 120 for clams in the low (0.03 mg l^{-1}) concentration of oil was significantly greater than that for the controls, but the condition indices for clams in 0.3 and 3.0 mg l^{-1} were lower than controls (Table 1). The higher condition index at the low oil concentration was due to a decrease in the rate of shell growth during oil exposure (Table 1). Shell

deposition in *Macoma balthica* begins with the formation of the periostracum, composed of tanned protein (Bubel, 1973). The quinone tanning hypothesized for periostracum formation is similar to the process of byssal thread formation in mussels (Hillman, 1961), which is adversely affected by oil exposure (Swedmark *et al.*, 1973; Dunning and Major, 1974). This process appears to be particularly sensitive to petroleum exposure, as it was one of the only two parameters which showed a significant difference from controls at the lowest level of oil exposure.

A question raised by the present experiment is why there was a decrease in dry weight relative to wet tissue weight in the clams exposed to the two higher oil concentrations. It is reasonable to conclude that as the clams use up their energy reserves, their dry weight will decrease. The controls displayed such a decrease in dry weight. Yet their dry to wet weight ratio remained at around 17.5% (Fig. 3). The clams in 3.0 mg l^{-1}, however, lost dry weight faster than wet weight, showing a value of less than 14% by Day 180. Such a relative increase in wet weight to dry weight might indicate that the clams are becoming osmotically unbalanced.

Clams exposed to the higher oil levels (0.3 and 3.0 mg l^{-1}) showed many signs of stress, probably as a result of a negative energy balance (Table 4). Losses in dry weight, decreases in shell growth, resorption of gametes, and decreases in carbohydrate content, all indicate energy utilization rather than storage. This condition probably was a consequence of the observed decrease in feeding behaviour, but was compounded by the increased oxygen demand of a higher metabolic rate (Table 2). A reduction in filtering efficiency may also have contributed to the energy deficit, as petroleum products have been observed to affect cilia behaviour (Johnson, 1977).

Total oxygen consumption, which is the sum of the basal metabolic rate and the rate due to activity, increases in many organisms exposed to petroleum, but decreases have also been observed (Johnson, 1977; Stainken, 1978). Percy (1977) has hypothesized that decreases in oxygen consumption are the result of activity reduction which may mask a general increase in basal metabolic rate. To support this hypothesis, he has shown that whereas intact amphipods exposed to oil showed variable changes in respiration rate in comparison to controls, cell-free homogenates prepared from amphipods exposed to oil had a higher metabolic rate than cell-free homogenates of amphipods not exposed to oil. Since *Macoma balthica* from the present set of experiments were not being fed at the time of oxygen consumption measurement, the increase in metabolism after oil exposure probably represents a change in the basal metabolic rate. Increased oxygen demand could result from enzymatic degradation of petroleum constituents; however, bivalves seem to be generally lacking in aryl hydrocarbon hydroxylase (Varanasi and Malins, 1977). As Stainken (1978) has suggested, increased metabolic rate may be the result of oxidative phosphorylization uncoupling.

Tissue RNA content provides a rough estimate of relative sustained protein synthesis. The constancy of RNA values after Day 30 indicates that this activity was not altered appreciably by oil exposure. The general trend toward higher DNA concentrations indicates that the average cell size of the clams was decreasing for the "high" DNA group. However, since DNA content showed no significant difference between oil-exposed and control groups, this is probably a normal effect, perhaps related to the seasonality of food supply or spermatogenesis.

It was part of our hypothesis that exposure of marine organisms to oil could have serious effects on membrane structures, since the lipid bilayer of membranes and oil are lipophilic. If the oil were able to dissolve into the membranes of the clam, then enzyme functions which depended on an intact membrane would be impaired. Enzymes such as Na^+-K^+-ATPase (the sodium pump) are well known to require associated lipid for proper function (White *et al.*, 1973). It has also been shown that compounds found in crude oil are able to enter the membranes of fish (Roubal, 1974).

In the present study, we investigated enzyme activities that are associated with the plasma membranes; however, we did not find evidence of general membrane disruption caused by oil. Only the Mg^{++}-ATPase activity was significantly reduced by exposure to both 0.3 and 3.0 mg l^{-1} oil in seawater (Fig. 4). The Na^+-K^+-ATPase appeared to be unaffected by oil, and the 5′-nucleotidase and phosphodiesterase activities appeared to be increased slightly. It is likely that these enzymes occupy different sorts of environments in the membranes which are differentially affected by oil (Roubal, 1974). A decrease in the activity of an enzyme caused by exposure to oil could be a result of direct action on the enzyme, or a result of action at another level such as on synthesis or degradation of the enzyme, or even effects on the relative levels of modifiers of the enzyme. The series of experiments performed *in vitro* on crude homogenates from *Macoma balthica* showed that the Mg^{++}-ATPase activity is decreased after crude oil and other hydrocarbons are mixed with the homogenates (Table 3). This lends support to the idea that the oil can directly interfere with membrane-bound enzyme activity.

Most of the Mg^{++}-ATPase activity (90%) was associated with the membrane fraction, as determined by differential centrifugation. About 50% of this membrane activity was associated with the mitochondrial pellet (15,000 x *g* pellet of 1,600 x *g* supernatant). It is likely that half of the ATPase activity is from mitochondrial electron transport, but that the rest is associated with the plasma membrane fraction. Since we did not differentiate between membrane fractions in this experiment, it is unclear whether the decrease in Mg^{++}-ATPase activity is due to the oil's effect on mitochondrial or plasma membranes. If the ATPase in the mitochondria were affected, this might be a result of the effect of oil on membrane permeability causing dysfunction in coupling ionic and pH membrane potentials to ATP synthesis (Boyer *et al.*, 1977). It is

ssible that such interference would cause uncoupling oxidative phosphorylation, leading in turn to an rease in the respiration rate. On the other hand, the $^{++}$-ATPase in the plasma membrane may be instrumental in supplying the energy needed to generate the ary movement necessary for pumping water through clam over the labial palps and the gills. If this Pase activity were decreased by oil, we would expect ecrease in the filtering rate of the clams. The observed crease in the feeding activity of the clams may reflect h a decrease in the pumping rate.

In the laboratory, many clams survived although viously weakened by exposure to oil. The clams were t subjected to any of the stresses that normally occur their natural environment in the mud flats, such as anges in salinity, temperature, oxygen availability, and ve action. Therefore, it is probable that exposure of ms to oil under field conditions results in higher rtality. For example, when the tides recede from the d flats in Resurrection Bay, the salinity of the water maining on the flats is lowered due to the freshwater n-off. To compensate for a change in salinity, bivalves er the composition and amounts of their free amino ids (Gainey, 1978). If the clams are already in a akened state induced by oil exposure, such natural anges in their environment might cause more serious nsequences than would be envisaged based on the oratory data reported in this paper. Even in the sence of such synergistic effects, our experimental sults strongly suggest that chronic exposure to oil ncentrations as low as 0.03 mg l^{-1} leads to significant creases in the viability of *Macoma balthica* populations.

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